

PRODUCTION OF ETHANOL BY IMMOBILIZED *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

This research was conducted on the fermentation of ethanol by *Saccharomyces cerevisiae* using immobilization technique. *S. cerevisiae* which is also known as Baker's yeast is a single-celled eukaryote that is frequently used in scientific research. It is an attractive model organism due to the fact that its genome has been sequenced, its genetics are easily manipulated and very easy to maintain in the laboratory. Besides, immobilization has already been approved as useful method in maintaining high cell concentration within the reactor, increases the cell's ability to tolerate high ethanol concentration in the medium, allowing higher conversion yield and increased volumetric productivities. Therefore, the purpose of this study is to immobilize the yeast using various kind of supports (e.g: muslin cloth, membrane and sugarcane stalk) in batch processes to determine the best support for ethanol production. In this method, immobilized fermentation was carried out through shake flask seeding technique while the duration of fermentation process was fixed at 10 hours with sampling made at every 2 hours intervals. The germination of the stock and seed culture preparations were conducted followed by the immobilization technique in order to produce ethanol. Samples taken were analyzed for ethanol and glucose concentration, colony forming unit (CFU), biomass concentration, optical density and viewing of *S. cerevisiae* attachment on immobilized supports by Scanning Electron Microscope (SEM). From the result obtained, it showed that immobilized cloth was capable to increase cell optical density up to 1.602 after 10 hours of fermentation. Moreover, analysis performed by using biomass has revealed that immobilized cloth at 10 hours of fermentation gave the highest biomass concentration which is 39.64 gL⁻¹ and colonies of cells forming at 2.57 x 10⁸/mL of viable cells compared to other materials. Besides, its ethanol productivity was enhanced to 0.42 gL⁻¹h⁻¹, being over 9.6% higher than that observed in the batch culture (0.38 gL⁻¹h⁻¹). The consumption of glucose was improved by 6.9% (compared to batch operation), where nearly 67% of glucose conversion at 10 hours period of immobilized fermentation through muslin cloth. The increment might be associated with the altered metabolic functions in the immobilized cells. This alteration is attributed to the reduction of the diffusion path of the growth nutrient that enhanced the availability and promoted the growth of yeast, thus improving the catalytic conversion of glucose to ethanol. Therefore, the supported muslin cloth was proved to increase the number of cells and glucose inhibition is able to reduce with the utilization of immobilized *S. cerevisiae*, thus increasing ethanol production.

ABSTRAK

Kajian ini telah dijalankan ke atas penapaian etanol oleh *Saccharomyces cerevisiae* menggunakan teknik imobilisasi. *S. cerevisiae* yang juga dikenali sebagai yis roti adalah satu eukariot unisel yang sering digunakan dalam penyelidikan saintifik. Ia adalah satu organisma model yang menarik berdasarkan fakta bahawa genomnya yang telah disusun, genetik yang mudah dimanipulasi dan sangat mudah untuk dikekalkan di dalam makmal. Selain itu, kaedah imobilisasi telah diluluskan kerana ia berguna dalam mengekalkan kepekatan sel yang tinggi dalam reaktor, meningkatkan keupayaan sel untuk bertolak ansur dengan kepekatan etanol yang tinggi di dalam media, yang memberikan hasil penukaran yang lebih tinggi dan meningkatkan isipadu produktiviti. Oleh itu, tujuan kajian ini adalah untuk mengimobilisasikan yis dengan menggunakan pelbagai jenis sokongan (cth: kain kasa, membran dan batang tebu) dalam proses berkelompok bagi menentukan sokongan yang terbaik untuk pengeluaran etanol. Dalam kaedah ini, penapaian secara imobilisasi dilakukan melalui teknik pembenihan kelalang manakala tempoh proses penapaian telah ditetapkan pada 10 jam dengan pensampelan dibuat pada setiap selang 2 jam. Persediaan bagi stok dan kultur benih telah dijalankan secara imobilisasi untuk menghasilkan etanol. Sampel yang diambil telah dianalisis untuk kepekatan etanol dan glukosa, pembentukan koloni unit (CFU), kepekatan sel, ketumpatan optik dan paparan *S. cerevisiae* yang berada di atas sokongan imobilisasi dilihat menggunakan SEM. Daripada keputusan yang diperolehi, ia menunjukkan bahawa imobilisasi menggunakan kain kasa mampu untuk meningkatkan kepadatan sel optik sehingga 1.602 selepas 10 jam penapaian. Selain itu, analisis yang dijalankan menggunakan sel jisim telah mendedahkan bahawa kain setelah 10 jam penapaian memberikan kepekatan sel jisim yang tertinggi iaitu 39.64 gL⁻¹ dan pembentukan sel koloni pada 2.57 x 10⁸ / mL sel apabila di bandingkan dengan bahan imobilisasi yang lain. Tambahan lagi, produktiviti etanol telah dipertingkatkan kepada 0.42 gL⁻¹h⁻¹, menunjukkan 9.6% lebih tinggi daripada yang diperhatikan dalam kultur berkelompok (0.38 gL⁻¹h⁻¹). Penggunaan glukosa telah meningkat sebanyak 6.9% (berbanding dengan operasi kelompok), di mana hampir 67% daripada penukaran glukosa pada 10 jam tempoh penapaian secara imobilisasi adalah melalui kain kasa. Peningkatan ini boleh dikaitkan dengan fungsi metabolik yang berubah di dalam sel-sel imobilisasi. Perubahan ini adalah disebabkan oleh pengurangan laluan resapan nutrien pertumbuhan yang meningkatkan tahap kesediaan dan pertumbuhan yis, sekali gus menyebabkan pertambahan terhadap penukaran pemangkin glukosa kepada etanol. Oleh itu, sokongan kain kasa telah terbukti mampu meningkatkan jumlah sel dan perencatan glukosa mampu dikurangkan dengan penggunaan imobilisasi *S. cerevisiae*, oleh itu pengeluaran ethanol dapat ditingkatkan.

TABLE OF CONTENTS

SUPERVISOR'S DECLARATION	IV
STUDENT'S DECLARATION	V
<i>Dedication</i>	VI
ACKNOWLEDGEMENT	VII
ABSTRACT	VIII
ABSTRAK	IX
TABLE OF CONTENTS	X
LIST OF FIGURES	XII
LIST OF TABLES	XIV
LIST OF ABBREVIATIONS	XV
1 INTRODUCTION	1
1.1 <i>Introduction</i>	1
1.2 <i>Motivation and statement of problem</i>	3
1.3 <i>Objectives</i>	4
1.4 <i>Scope of this research</i>	4
1.5 <i>Main contribution of this work</i>	4
1.6 <i>Organization of the thesis</i>	5
2 LITERATURE REVIEW	6
2.1 <i>Overview</i>	6
2.2 <i>Introduction</i>	6
2.3 <i>Global Ethanol Demand Reviews</i>	7
2.4 <i>Current Ethanol Production Technology and Lignocellulosic Ethanol Biorefinery</i>	9
2.5 <i>Ethanol Producers</i>	10
2.6 <i>Yeast (Saccharomyces cerevisiae)</i>	13
2.7 <i>Immobilization</i>	15
2.7.1 <i>Importance of Immobilization</i>	15
2.8 <i>Effect of Immobilizations on Yeast Morphology and Physiology</i>	17
2.9 <i>Principle of Bacteria Growth</i>	18
2.9.1 <i>Lag phase</i>	20
2.9.2 <i>Exponential or Logarithmic (log) phase</i>	20
2.9.3 <i>Stationary phase</i>	20
2.9.4 <i>Decline or Death phase</i>	21
2.10 <i>Factors Affecting Ethanol Production</i>	21
2.11 <i>Effect of Ethanol Accumulation on the Growth of Yeast</i>	22
2.12 <i>Previous Work on Ethanol Production within Free and Immobilized Cells</i>	23
2.13 <i>Analysis Method for Ethanol Production</i>	25
2.14 <i>Summary</i>	26
3 MATERIALS AND METHODS	27
3.1 <i>Overview</i>	27

3.2	<i>Introduction</i>	27
3.3	<i>Materials</i>	28
3.3.1	<i>Chemicals</i>	28
3.3.2	<i>Immobilized Cells and Supports</i>	28
3.4	<i>Media preparation</i>	28
3.4.1	<i>Agar Medium for S. cerevisiae</i>	28
3.4.2	<i>Growth Medium for S. cerevisiae</i>	29
3.4.3	<i>Production Media for Ethanol from S. cerevisiae (Batch Fermentation)</i>	30
3.4.4	<i>Phosphate Buffer Saline (PBS) Solution</i>	30
3.4.5	<i>Saline Solution</i>	31
3.5	<i>Preservation of Stock Culture</i>	31
3.6	<i>Culture Preparation</i>	32
3.7	<i>Immobilization Procedure</i>	33
3.7.1	<i>Shake Flask Seeding Technique</i>	33
3.8	<i>Analysis of data</i>	34
3.8.1	<i>Cells Optical Density, Biomass and Colony Forming Unit (CFU)</i>	34
3.8.2	<i>Dinitrosalicylic Acid (DNS) Method</i>	34
3.8.3	<i>Gas Chromatography (GC-FID)</i>	35
3.8.4	<i>Scanning Electron Microscope (SEM)</i>	35
4	PRODUCTION OF ETHANOL THROUGH IMMOBILIZED SACCHAROMYCES CEREVISIAE	36
4.1	<i>Overview</i>	36
4.2	<i>Introduction</i>	36
4.3	<i>Kinetic Growth Profile of S. cerevisiae</i>	36
4.4	<i>Effect of Immobilization on Cell Optical Density, Biomass Concentration and Colony Forming Unit (CFU)</i>	38
4.5	<i>Glucose Utilization and Ethanol Production by Immobilized S. cerevisiae</i>	42
4.6	<i>Improvement of Yield against Batch Fermentation</i>	44
4.7	<i>Morphology of Immobilized Cells within Muslin Cloth</i>	45
4.8	<i>Summary</i>	46
5	CONCLUSION	47
5.1	<i>Conclusion</i>	47
5.2	<i>Recommendation</i>	47
6	REFERENCES	48
7	APPENDICES	53

LIST OF FIGURES

Figure 2-1: U.S Energy consumptions in 2009 (Biofuels and Global energy, 2011).....	8
Figure 2-2: Evolution of the variable cost of ethanol production from maize treated by the dry-grind process and hypothetical S-curve in technological improvement of lignocellulosic ethanol.	9
Figure 2-3: The shape of <i>S. cerevisiae</i> a) patented by Fleischmann's (Thorp, 2009) and b) examined under Staining Electron microscope (SEM) using 650x magnification with scale bar of 20 μm (Konig <i>et al.</i> , 2009).	13
Figure 2-4: Schematic diagram of <i>S.cerevisiae</i> (Wan Salwanis, 2013).	13
Figure 2-5: Basic method of cell immobilization. (Lacroix, 1996)	16
Figure 2-6: General kinetic growth curve of bacteria (Benjamin, 2004).	19
Figure 2-7: Potential environmental stresses on <i>S. cerevisiae</i> during ethanol fermentation in both free and immobilized cells (Bai <i>et al.</i> , 2008).	21
Figure 3-1: Flow chart process of experiment	27
Figure 3-2: Preparation of a) nutrient agar and b) agar plates.	29
Figure 3-3: Preparation of a) growth medium for <i>S. cerevisiae</i> b) seed culture.	29
Figure 3-4: Preparation of a) media for ethanol production with 150 gL^{-1} amount of glucose b) medium containing immobilized supports.	30
Figure 3-5: Preparation of a) PBS solution for b) bacterial suspension medium.	31
Figure 3-6: Preparation of a) saline solution for cell washing and b) removal of cell debris.	31
Figure 3-7: Preparation and preservation of stock culture in sterilized glycerol solution.	32
Figure 3-8: Germination of yeast cells on agar medium from stock culture	33
Figure 3-9: Preparation of production media for a) batch fermentation and b) immobilized fermentation.	33
Figure 4-1: Growth profile of <i>S. cerevisiae</i> and its biomass concentration for 10 hours fermentation fixed at 30°C and 200 rpm	37
Figure 4-2:(a) Cells OD at 600 nm and (b) biomass concentration using different types of immobilized supports for 10 hours fermentation fixed at 30°C and 200 rpm	39
Figure 4-3: Observation on immobilized supports after 10 hours fermentation. (Process conditions: pH 5.0; 30 °C; 200 rpm; initial glucose concentration 150 gL^{-1})	40
Figure 4-4: The number of immobilized cells during 10 hours of fermentation by <i>S. cerevisiae</i> with various types of immobilized supports. (Process conditions: pH 5.0; 30°C; 200 rpm; initial glucose concentration 150 gL^{-1})	40
Figure 4-5: Profile of glucose and ethanol concentration by <i>S. cerevisiae</i> immobilized in various supports for 10 hours fixed at 30°C and 200 rpm	43
Figure 4-6: Optical microphotograph of <i>Saccharomyces cerevisiae</i> cells at a) magnification of 1000x b) magnification of 2000x that immobilised into muslin cloth after 10 hours of fermentation.	46

Figure 7-1: Graph of DNS standard curve	53
Figure 7-2: Profile of glucose consumption relative to 10 hours of fermentation.....	54
Figure 7-3: Graph of ethanol concentration standard curve.....	55
Figure 7-4: Graph of peak area which respect to ethanol concentration for different immobilized supports.....	55

LIST OF TABLES

Table 2-1: World fuel ethanol production by certain country (Urbanchuk, 2009).....	8
Table 2-2: Lists of the ethanol producer comprised with its advantages and disadvantages	12
Table 3-1: Composition of PBS solution.....	30
Table 4-1: Data on the attachment of cells on immobilized supports after 10 hours of fermentation fixed at 30 ⁰ C and 200 rpm	39
Table 4-2: Data on the yield of production depending on different immobilized supports after 10 hours of fermentation fixed at 30 ⁰ C and 200 rpm.....	45
Table 7-1: Data of optical density obtained in 10 hours of fermentation fixed at 30 ⁰ C and 200 rpm	53
Table 7-2: Data of cell dry weight obtained in 10 hours of fermentation fixed at 30 ⁰ C and 200 rpm	53
Table 7-3: Data of Colony Forming Unit (CFU) obtained in 10 hours of fermentation fixed at 30 ⁰ C and 200 rpm.....	54
Table 7-4: Data of standard curve for DNS method obtained in 10 hours fermentation fixed at 30 ⁰ C and 200 rpm.....	54
Table 7-5: Data of DNS method analysis for samples obtained in 10 hours fermentation fixed at 30 ⁰ C and 200 rpm.....	55
Table 7-6: Data of ethanol concentration for standard and fermentation samples obtained in 10 hours fermentation fixed at 30 ⁰ C and 200 rpm.....	56
Table 7-7: Data of optical density for bacterial suspension solution before and after the immobilization method	57

LIST OF ABBREVIATIONS

$\% w/v$	percentage of weight per volume
$\%v/v$	percentage of volume per volume
<i>CDW</i>	dry cell weight
<i>CFU</i>	colony forming unit (cells/mL)
C_P	concentration of product (ethanol)
C_S	concentration of substrate (glucose)
C_X	concentration of biomass (cells)
<i>DNS</i>	dinitrosalicylic
<i>G</i>	glucose
<i>GC</i>	gas chromatography
H_3PO_4	phosphoric acid
<i>HCl</i>	hydrochloric acid
<i>O/W</i>	oil in water emulsion
O_2	oxygen
<i>OD</i>	optical density
<i>P</i>	peptone
<i>PBS</i>	phosphate buffered saline
<i>SEM</i>	scanning electron microscopy
<i>SF</i>	shake flask
<i>V</i>	volume (cm^3/mL)
<i>W</i>	weight of biomass (g)
<i>W/O</i>	water in oil emulsion
<i>YE</i>	yeast extract
$Y_{P/S}$	yield of product form over substrate consumed
$Y_{P/X}$	yield of product form over biomass produced
<i>YPG</i>	media containing yeast extract, peptone and glucose.
$Y_{X/S}$	yield of biomass produced over substrate consumed
μ_{max}	specific maximum growth (hr^{-1})

1 INTRODUCTION

1.1 Introduction

Ethylene hydration process is being used in the early days to produce commercial ethanol, derived from fossil fuel by using phosphoric acid as a catalyst. However, nowadays, 90% of the total ethanol is derived from sugar through microbial action (fermentation process). Three major steps that involved in the process are: i) the production of simple sugar from the main feedstock via enzyme or chemical hydrolysis, ii) conversion of sugar to ethanol by microorganisms, and iii) a treatment process to separate ethanol from the fermentation broth (Balat *et al.*, 2008). Conventional batch or fed-batch modes are usually used to carry out the fermentation operation by utilising various feedstocks such as sugarcane, corn, wheat, beet root and tapioca. But currently, ethanol is frequently produced by fermentation when certain species of yeast (*Saccharomyces cerevisiae*) metabolize sugar in the absence of oxygen they will produce ethanol and carbon dioxide (Anxo *et al.*, 2008).

Since ethanol has been chosen as an important industrial chemical with emerging potential as a biofuel to replace fossil fuels, the full attention has been paid especially to the economics and energy consumption factor (Demirbas, 2006). Due to its characteristic of being friendly to environment, ethanol has been labelled as the one of the most advanced liquid fuels in the world. Ethanol (C_2H_5OH) is a monohydric primary alcohol which also known as ethyl alcohol. It is the second member of the aliphatic alcohol series consist of clear, colourless liquid with characteristic odour and taste of pleasant smell which commonly called grain alcohol or simply alcohol. In dilute aqueous solution, it has a sweet flavour, but in more concentrated solutions it has a burning taste (Patil 1991). It is categorized in a group of chemical compounds whose molecules contain an OH group, bonded to a carbon atom (Kaur & Kocher, 2002).

Ethanol is particularly useful in industrial applications because of its relatively high affinity for both water and organic compounds (Anxo *et al.*, 2008). Beyond its use as an automotive fuel, ethanol can be produced for use in a various applications in industries including chemistry, pharmaceutical and food industries as a form of raw materials, solvents and fuel. One of the largest users of industrial ethanol is including

personal care products industry especially in many hand sanitizers, soaps, and shampoos. Besides, it also gave major contribution in processing antibiotics, vaccines, tablets, pills, and vitamins due to its properties that act as a solvent for the pharmaceutical industry.

Various processes have been developed for ethanol production but worldwide demand of ethanol is generally satisfied by biotechnological fermentation process (Walker *et al.*, 1990). Recently, cell immobilization techniques have become increasingly important and are being successfully applied in production of ethanol through fermentation process (Reddy *et al.*, 2008). These procedures frequently improve catalyst stability and the immobilized cells can be concentrated to higher densities within the immobilization support that is impossible in normal suspension cultures, resulting in potentially high reactor productivities. However, this technique has also shown a few disadvantages in terms of the stability and fermentation performance. Several problems have been identified, which are mainly due to the longer operating time and cell losses. The poor mechanical strength of the support enhances cell leakage during prolonged operation. Due to the problems occurred, applying the laboratory works into the real industrial process is almost impossible if the problems have not been resolved.

Therefore, the aim of this study is to investigate the effect of immobilized yeast (*Saccharomyces cerevisiae*) on the production of ethanol using various kind of supports (e.g: muslin cloth, sugarcane stalk and membrane) in batch processes thus, determine the best support for ethanol production. The ethanol produced is proportional to the growth of yeast, so preparing suitable housing with unlimited spaces that permit the proliferation of yeast is crucial for a long term continuous operation. Immobilization technique and the types of supports will be thoroughly studied.

1.2 Motivation and statement of problem

Presently, fossil fuel is the main source of world energy production. But, the depletion of fossil fuels due to the environmental problems has become a serious energy crisis to the world. Therefore, biomass has been utilized for the production of friendly biofuels such as ethanol. Since the ethanol market has developed fantastically, the diversification of processes in terms of operating mode, feedstock, and searching for an effective and efficient microorganism has been extensively studied. The major research and development challenges for ethanol production from biomass basically involve several factors such as the improvement of quality and feedstock for its production and techno-economic along with environmental assessment by minimising the negative impact on the environment relating to agricultural issues on land and wastes (Gnansounou, 2010). Unfortunately, ethanol fermentation nowadays encountering more disadvantages rather than advantages such as the cost of production is too high, more energy is required, production of the process is too slow and the product obtained is impure so additional pretreatment are needed. This problem occurs will lead to the time consuming factor and decrease the product yield. Although continuous process helps to reduce the cost and operation time by terminating some of the production steps in the batch operation, the washout effect and contamination resulting from the long operation time reduces its effectiveness. This kind of problem can be improved by applying the immobilization technique. It is a technique where the microorganisms were grown in insoluble matrix (support) which permits the flow of liquid in and out but retains the cells within its capsules. Due to this technique, the cell's ability can be increased to tolerate high ethanol concentration in the fermentation medium, allowing higher conversion yield and increased volumetric productivities (Qi *et al.*, 2006). Besides, the immobilization technique allows the reuse of cells, so the operating cost can be reduced. Therefore, ethanol production by immobilized cells has been intensively investigated during last few years because this technique showed certain technical and economic advantages over free cells system that can be more profitable rather than past years of operation used.

1.3 Objectives

The objectives of this study are to investigate the effect of immobilized yeast (*Saccharomyces cerevisiae*) on the production of ethanol using various kind of supports (e.g: muslin cloth, sugarcane stalk and membrane) in batch processes and to determine the best support for the production thus reducing the effect of glucose inhibition and increasing the ethanol production.

1.4 Scope of this research

In this research study, the scopes function as a guideline to achieve the objectives. The study has been divided into several scopes which are:

- 1.4.1 To perform growth profile of ethanol production through submerged fermentation using free cell *Saccharomyces cerevisiae* in batch processes.
- 1.4.2 To carry out fermentation process using immobilized cells on various kind of supports (e.g: muslin cloth, membrane and sugarcane stalk).
- 1.4.3 To study the effects of glucose concentration on the ethanol production using the best support in (1.4.2).

1.5 Main contribution of this work

This research was capable to investigate the effect of various kinds of supports (eg: muslin cloth, membrane and sugarcane stalks) on the productivity of ethanol production. According to the previous researchers, the production of ethanol by immobilizing organism had been demonstrated to have greater advantages over a suspended culture of free cells of *S. cerevisiae*. Although it is consider as a time consuming technique that needed additional equipment and materials, it is one of the best method since it enable to contain the cells within its capsulation, thus avoiding the cells to be present in products, therefore an additional step of purification can be discarded. Hence, ethanol production was effectively enhanced to yield the maximum amount of product through immobilized supports if compared to batch operation. Nevertheless, it was able to help people effectively improve their knowledge and understanding on the immobilized fermentation of ethanol.

1.6 Organization of the thesis

The structure of the thesis was outlined as follow:

Chapter 2 provided a description on the ethanol characteristic and its application. Moreover, the benefits of using *S. cerevisiae* as the free immobilized cells and also the advantages rather than disadvantages of immobilization technique were explained. This chapter also provided a brief discussion on ethanol producers, characteristic of yeast (*Saccharomyces cerevisiae*) and its growth profile along with the future demand of ethanol production. A summary of the previous reported experimental work on ethanol fermentation process through immobilization technique was also presented.

Chapter 3 gave a review on the chemicals, materials and all the experimental works employed for fermentation stage.

Chapter 4 was devoted on the result and observation obtained from the analyzes performed with samples taken from the fermentation process to study the effect of immobilized yeast (*Saccharomyces cerevisiae*) on the production of ethanol using various kind of supports (e.g: muslin cloth, sugarcane stalk and membrane). Therefore, the analysis data that was conducted were through direct and indirect methods including determination of glucose concentration by DNS method and ethanol concentration using Gas Chromatography, colony forming unit (CFU), biomass concentration, optical density and viewing of *S. cerevisiae* attachment on immobilized supports by SEM was demonstrated in this chapter. Besides, the kinetic growth profile of the immobilized cells has also been illustrated to examine the trend of growth curve of the yeast cells.

Chapter 5 was concluded the overall study result and observation related to the immobilized fermentation in terms of glucose and ethanol concentration, its yield and productivity. Besides, the best support to immobilize the *S. cerevisiae* was determined by performing analysis on the cell absorbance, biomass concentration and the cells forming unit. The recommendation regarding the best support for the immobilize cells was also provided.

2 LITERATURE REVIEW

2.1 Overview

This research was conducted to investigate the production of ethanol through immobilized fermentation of *S. cerevisiae*. Ethanol has been extensively studied as a kind of renewable resource due to its characteristic that burns more cleanly in air than petroleum, producing less carbon (soot) and carbon monoxide. Ethanol-fuelled vehicles produce lower carbon monoxide and carbon dioxide emissions, and the same or lower levels of hydrocarbon and oxides of nitrogen emissions. Therefore, it is one of the best tools we have to fight air pollution from vehicles. There is no fuel available that matches the ethanol's ability to improve overall environmental quality compared to gasoline. From its biodegradable nature to reductions in greenhouse gas and tailpipe emissions, ethanol provides a tool to address environmental concerns without requiring an entirely new way for goods and people to get from one place to another. Hence, the best method to be used that give the maximum yield of ethanol production with the minimized disadvantages is the immobilization technique. Although there are several disadvantages have been mentioned by the previous researchers, but from the positive perspective, immobilization offers more advantages compared to other techniques.

2.2 Introduction

A microbial fermentation for ethanol production by using immobilized fermentation method was intensively studied previously. However, there are only several researchers use immobilization as the preferred method to be carried out probably due to time constraints and additional cost is needed. Therefore, most of the research studies were focussing on the production of ethanol by using batch and continuous culture. Hence, previous work on production of ethanol was explained below. Besides, the detail information about world ethanol demand, ethanol producers, yeast *Saccharomyces cerevisiae*, general growth profile possess by a bacteria and immobilization technique were described as below.

2.3 Global Ethanol Demand Reviews

Nowadays, the prime focus of ethanol production throughout the world has led to the continuous demand for its energy consumption factor. Due to the problem that facing by the current energy produced from fossil fuel, it has created debates among the experts since it can give negative impact on the environment and the emerging doubts concerning its sustainability for the future. Therefore, many countries have started to use ethanol as their main energy source that can be applied in various kind of industrial and daily application. One of the largest country that consume and produce ethanol as their main energy source is United States.

In the U.S., the Energy Independence and Security Act of 2007 requires that 36 billion gallons of renewable fuels, largely ethanol, be used in the nation's motor fuel supply by 2022. According to the Renewable Fuels Association, the U.S. ethanol industry has current capacity to produce more than 8.5 billion gallons of ethanol and an additional 5.1 billion of new capacity are under construction and will come on-line within the next several years. 20.4 billion gallons of ethanol production expands the quantity of gasoline available to consumers around the world (Urbanchuk, 2008). Although the environmental costs associated with producing ethanol are significant, it have been ignored by most investigators in terms of energy and economics (Pimentel *et al.*, 2007). Since ethanol has lower energy content than gasoline, there is not a one-to-one substitution of ethanol for gasoline. Reflecting this, if ethanol were not available, the world's oil refiners would have to "find" an additional 13.4 billion gallons (320 million barrels) of gasoline to make up the shortfall.

On the other hand, according to the EIA and referring to Figure 2-1, renewable fuels in the US in 2009 make up 1.6% of the energy used, with 13 billion gallons of ethanol produced in last year of 2008. For better or for worse, ethanol is part of our future. Not only has the government mandated a 36 billion gallon target by the EPA for 2022, but the EPA has recently approved the use of E-15 blended gasoline (15% ethanol) for cars that are as old as 2001. Essentially, the majority of cars and trucks on the road can now run on E-15, which is better for the environment as burning ethanol produces less greenhouse gas emissions, but the downside is you need more of it, as ethanol contains less energy than gasoline (Biofuels and Global energy, 2011).

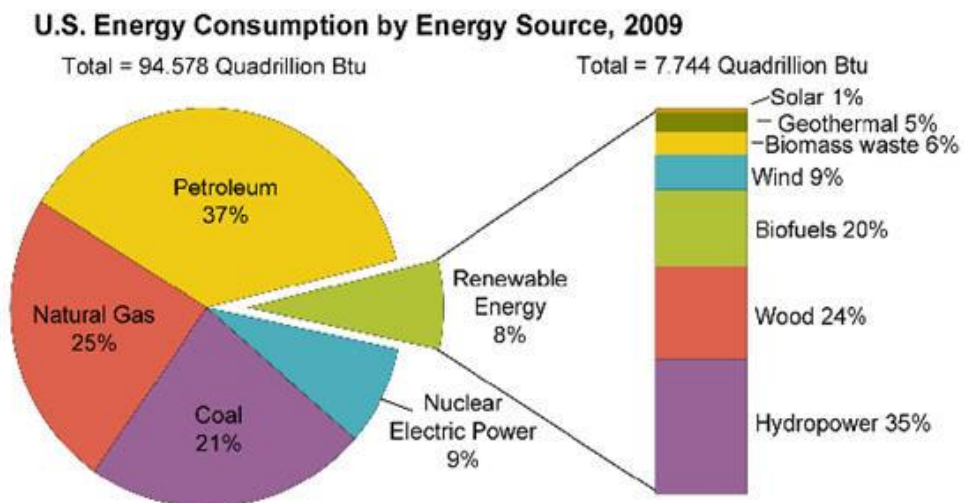


Figure 2-1: U.S Energy consumptions in 2009 (Biofuels and Global energy, 2011)

From the overall production in all over the world, Table 2-1 reported that ethanol production is said to reach 13.1 billion gallons in 2007 and is projected to total nearly 20 billion gallons in year 2009. This represents a near doubling of ethanol production in just five years. More than 7 countries reported annual production in excess of 100 million gallons with the two largest producers which are the U.S. and Brazil that accounting for nearly 78 percent of global ethanol production. The growth in global ethanol production has been the result of increased interest in biofuels as a result of sharply rising oil and gasoline prices. Global ethanol production is expected to continue expanding as world crude oil prices increase to new record levels and remain high (Urbanchuk, 2009).

Table 2-1: World fuel ethanol production by certain country (Urbanchuk, 2009)

World Fuel Ethanol Production by Country (Millions Gallons)			
Country	2007	2008	2009
USA	6,499	9,000	10,600
Brazil	5,019	6,472	6,578
Europe	570	734	1,040
China	486	502	542
Canada	211	238	291
Thailand	79	90	435
Colombia	75	79	83
India	53	66	92
Australia	26	26	57
Other	82	128	247
WORLD	13,101	17,335	19,965

2.4 Current Ethanol Production Technology and Lignocellulosic Ethanol Biorefinery

Virtually all biotechnological ethanol fuel currently manufactured at the industrial scale is produced through fermentation of relatively easily accessible sugars by the yeast *S. cerevisiae*. Beyond the relatively limited level of worldwide investment made to this date to finance industrial biotechnology projects (Kircher, 2006), it is the various robustness of the cell and process economics limitations that currently restrict the profitability of lignocellulosic ethanol, and thus still to this date hinders the financing and development of efficient value chains to convert lignocellulose into commodity chemicals.

Most of the biotechnological ethanol currently derived from maize is produced from the dry grind process, a process which was designed to maximize capital return per hectolitre of maize-derived ethanol, as opposed to extracting the full value of the maize kernel. In nominal terms, the variable costs of dry grind ethanol have exponentially decreased from approximately 0.925 \$ L⁻¹ in the 1980s to a value that remained essentially unchanged between 1998 (0.251 \$ L⁻¹) and 2002 (0.253 \$ L⁻¹) (Shapouri, 2005). When plotted versus time, the ethanol manufacturing variable cost curve reveals that the present yeast-based technology has already reached its productivity limit (Figure 2-2), since its shape suggests that the current strategy of incremental improvements has reached a threshold of marginal return on investments.

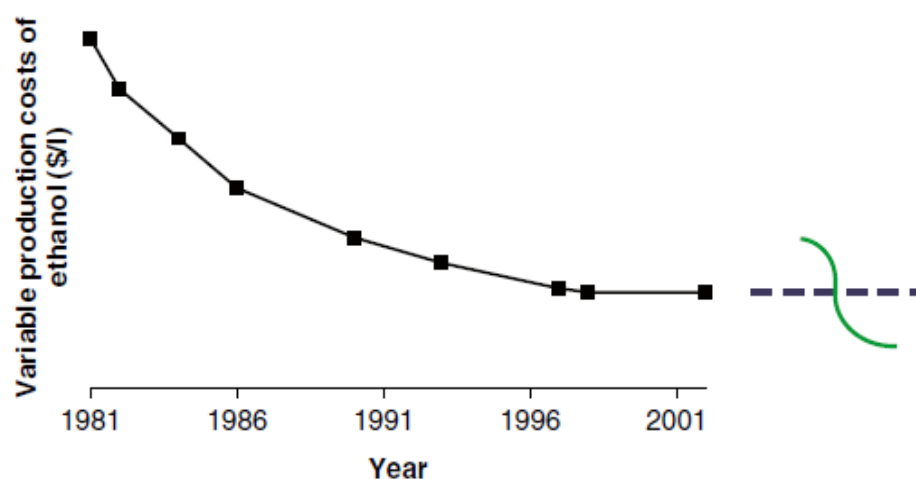


Figure 2-2: Evolution of the variable cost of ethanol production from maize treated by the dry-grind process and hypothetical S-curve in technological improvement of lignocellulosic ethanol.

Interestingly, over recent years dry mills have already experienced reduced energy, labour, and maintenance inputs, in addition to economies of scale (Shapouri, 2006). As a result, these 20 year data suggest that a novel technological discontinuity is now needed in order to further improve the economic performance of ethanol manufacturing. Considering that the net costs of raw materials constitute the most significant cost of biotechnological ethanol, it appears now increasingly urgent to implement alternative raw materials, such as lignocellulosic materials, and to generate by-products of higher value and deeper market than, for example, Distillers Dried Grains with Soluble. All these various components can be captured by the materialization of the biorefinery concept, which closely mimics the petrochemical refinery economic model.

Although yeast based technologies might to this date represent the optimal solution for the batch process conversion into fuel ethanol, this technological approach might not always be the most appropriate. For example, it cannot be excluded that a compact plant operating with growth-arrested microbial cells might be more efficient at exploiting the resources of agricultural or industrial environments where land is scarce, of low fertility or where infrastructures are insufficiently developed. Consequently, only a portfolio of technological options would give the flexibility that is necessary to enable the optimal use of such numerous raw material opportunities, and thus constitutes the most adequate response to address the challenges of an imminent energy crisis.

2.5 Ethanol Producers

Ethanol can be produced by fermentation of sugars from various waste agricultural materials. Whichever system for ethanol production is chosen, the attention must be paid to the overall economics and energy consumption. The economic evaluation of different materials for ethanol production was thoroughly studied previously (Meo, 1984; Maiorella *et al.*, 1984; Greg and Saddler 1995).

In recent years, metabolic engineering for microorganisms used in fuel ethanol production has shown significant progress. The most commonly used ethanol producer is *Saccharomyces cerevisiae* but recently, microorganisms such as *Zymomonas mobilis*, *Escherichia coli* and a few others also have been targeted through metabolic engineering for ethanol production as illustrated in Table 2-2 (Jeffries *et al.*, 2004).

Saccharomyces cerevisiae, the most widely utilised in industry, is considered the best organism because of its high yield and robustness. However, its application was found to be limited because of its inability to utilise C5 sugars. This is necessary since feedstock for the ethanol production is undergoing a shift from using food crops (corn and sugar cane) to cheaper lignocellulosic materials, therefore a more robust strain is required to ferment the C5 and C6 sugars that are produced from cellulose and hemicellulose hydrolysis. Apart from this, the preferred strain must be able to withstand the toxicity effect arising from lignin degradation (Jamai *et al.*, 2001).

Efficient ethanol production requires a rapid fermentation leading to high ethanol concentrations; therefore a yeast strain must have a good specific growth rate and good specific ethanol production rate at high osmotic activities and ethanol concentration. Therefore, *Saccharomyces cerevisiae* is considered to be the best organism due to its high yield and robustness. Traditionally, baker's yeast (*Saccharomyces cerevisiae*), has long been used in the brewery industry to produce ethanol from hexoses (six-carbon sugars). Due to the complex nature of the carbohydrates present in lignocellulosic biomass, a significant amount of xylose and arabinose (five-carbon sugars derived from the hemicellulose portion of the lignocellulose) is also present in the hydrolysate. Therefore, the ability of the fermenting microorganisms to use the whole range of sugars available from the hydrolysate is vital in the fermentation process. Yeast cells are especially attractive for cellulosic ethanol processes because they have been used in biotechnology for hundreds of years, are tolerant to high ethanol and inhibitor concentrations and can grow at low pH values to reduce bacterial contamination.

There are some basic requirements that must be fulfilled for selecting a microorganism as an ethanol producer, such as (Dien *et al.*, 2003):

- ❖ Ethanol yield must be more than 90% of theoretical
- ❖ Ethanol tolerance must exceed $>40 \text{ gL}^{-1}$
- ❖ Ethanol Productivity $> 1 \text{ gL}^{-1}\text{h}^{-1}$
- ❖ Robust, require inexpensive medium formulation with various substrate.
- ❖ High resistance to inhibitors and osmotic stress (high sugar concentration)
- ❖ Able to grow in acidic environment and higher temperatures
- ❖ Elimination of by-product formation (Kunz, 2008)

Table 2-2: Lists of the ethanol producer comprised with its advantages and disadvantages

Microorganism	Advantages	Disadvantages	References
<i>Saccharomyces cerevisiae</i>	<ul style="list-style-type: none"> • Considerable high ethanol yield • Fast growth • Robust • Stable • High tolerance to inhibitor 	<ul style="list-style-type: none"> • Growth related production • Susceptible to high ethanol concentration • Limited substrate • Contamination 	Karagoz <i>et al.</i> , (2008); Kunz (2008); Balat <i>et al.</i> , (2008)
<i>Zymomonas mobilis</i>	<ul style="list-style-type: none"> • Produce less biomass • Higher yield (5-10% more ethanol per g glucose) • High tolerance towards ethanol (up to 120 gL⁻¹). • High productivity • Safe 	<ul style="list-style-type: none"> • Ferments only glucose, sucrose and fructose • Low tolerance to acetic acid • Unstable 	Amin and Verachtert, (1982); Delgenes <i>et al.</i> , (1996); Kesava <i>et al.</i> , (1996); Dien <i>et al.</i> , (2003).
<i>Klebsiella oxytoca</i>	<ul style="list-style-type: none"> • Able to grow in acidic pH and higher temperature. • Utilizes wide range of sugars (C5-C6) 	<ul style="list-style-type: none"> • Yields many by-products • Low ethanol yield 	Dien <i>et al.</i> , (2003)
Recombinant <i>Escherichia coli</i>	<ul style="list-style-type: none"> • Able to ferment various sugars • Produce less biomass compare to <i>S. cerevisiae</i> 	<ul style="list-style-type: none"> • Low tolerance to pH (6.0-8.0) • Less hardy cultures • Problems with biomass and public perceptions 	Dien <i>et al.</i> , (2003).
<i>Candida tropicalis</i>	<ul style="list-style-type: none"> • Thermotolerant • Able to ferment various carbon sources (alcohols and sugar) • Ability to tolerate lignin like-polyphenols 	<ul style="list-style-type: none"> • Lower productivity compared to <i>S. cerevisiae</i>. • Lower tolerance towards ethanol • Slow adaptation to anaerobic condition 	Jamai <i>et al.</i> , (2001); De Deken (1966).
<i>Pichia Stipitis</i>	<ul style="list-style-type: none"> • Able to ferment C5-carbon source 	<ul style="list-style-type: none"> • Low yield 	Balat <i>et al.</i> , (2008)

2.6 Yeast (*Saccharomyces cerevisiae*)

S. cerevisiae was adopted as a model system for laboratory study in the 1930s, as investigators developed genetic tools to understand its life cycle and differentiation (Hall and Linder, 1993). Extensive studies also have been carried out on the fermentation process of ethanol by others organisms, however, *S. cerevisiae* remained the organism of choice, which is the same species used for bread making and some wines or beers (Walker *et al.*, 1990; Converti *et al.*, 2003; Moreira *et al.*, 2005). Figure 2-3 demonstrates the characteristic of this kind of yeast that was examined under a Staining Electron microscope with higher magnification and resolution. The schematic diagram of *S. cerevisiae* is also presented in Figure 2-4.

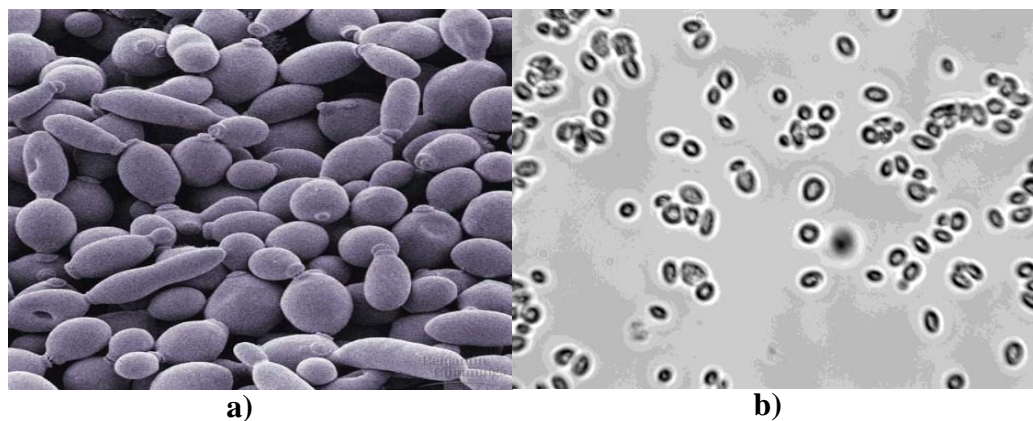


Figure 2-3: The shape of *S. cerevisiae* a) patented by Fleischmann's (Thorp, 2009) and b) examined under Staining Electron microscope (SEM) using 650x magnification with scale bar of 20 μm (Konig *et al.*, 2009).

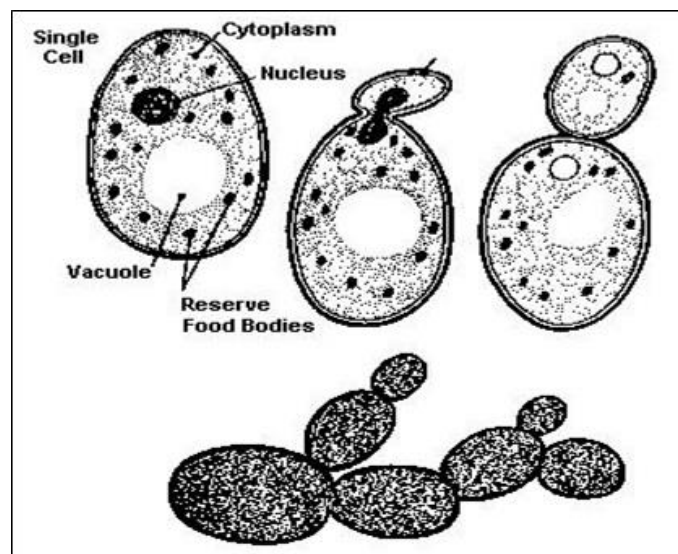


Figure 2-4: Schematic diagram of *S.cerevisiae* (Wan Salwanis, 2013)

S. cerevisiae is a unicellular microorganism which belongs to the fungi group. Its typical shapes are either spherical or oval, with clear internal cell structures. Observation under electron microscope, shows that *S. cerevisiae* consists of vacuole, mitochondria, cytoplasm and nucleus (Werner-Washburne *et al.*, 1993). Normally, most the size of yeasts can vary from 3-5 μm in diameter, although some can reach 7 μm . *S. cerevisiae* is commonly grow by budding, but very rarely it can also multiply by binary fission for instance as in *Saccharomyces pombe*. Due to the surface of the yeast is normally negatively charged, thus it can be easily immobilized on solid surfaces of opposite charge. Generally, yeast is hydrophobic therefore it prefers hydrophobic surfaces, and attaches weakly to hydrophilic surfaces such as glass (Fortman *et al.*, 2008).

The advantages of *S. cerevisiae* for industrial ethanol production include its long history of safe use, a high ethanol tolerance (up to 15% ethanol), and high final ethanol concentration (10–12%). Moreover, its sugar conversion ratios are close to the theoretical maximum (95%) and its tolerance of low pH helps prevent the growth of spoilage organisms. The robustness of yeast-based processes is limited by the sensitivity of *S. cerevisiae* to temperatures higher than 35°C, to the contamination of lactic acid bacteria, and to glucose repression phenomena that regulate its central metabolism. Moreover, the growth of *S. cerevisiae* is inhibited by a variety of compounds generated during the saccharification of lignocellulosic material and it cannot ferment xylose or arabinose. These two pentoses represent a significant portion, respectively 20% and 5% of the sugars in lignocellulosic biomass (Aristidou and Penttilä, 2000). Furthermore, actively growing yeasts produce ethanol up to 33 times faster than stationary cells (Bellissimi & Ingledew, 2005) preventing the use of growth-arrested yeasts.

Although *S. cerevisiae* was successfully employed in industry, further improvement is still desirable with respect to increased productivity, stability and tolerance towards stressors. Improvements are still evolving and relying on the random mutagenesis, classical breeding and genetic crossing of two strains and a successful strain with the previously mentioned qualities will be selected for the optimisation (Kunz, 2008). Recent studies were also directed to the thermotolerant strains, because high temperature is needed to withstand the requirement of high temperature (45-50°C) for the saccharification process in the simultaneous saccharification and fermentation (SSF) process (Edgardo *et al.*, 2008; Ylilervo *et al.*, 2011).

2.7 Immobilization

2.7.1 Importance of Immobilization

Immobilized cell system has been suggested as an effective means for improving ethanol fermentation, since it is possible to increase the concentration of cells within the reactor volume, hence increasing the product formation and process productivity, thus minimizing production costs (Santos *et al.*, 2008). The immobilization of cells leads to higher cell densities with consequent increases in reaction rates and productivity. As a result, shorter residence time and smaller reactor size can be employed. For industrial purposes, an important choice criterion is the carrier cost which, combined with the interest in by-products recycling, has been leading to an increasing search for cheap and available potential cell carriers (Santos *et al.*, 2008).

Immobilized microbial cell systems offer advantages over cell suspension systems in terms of ethanol productivity and the stability of cell activity (Sho *et al.*, 2001). Cell immobilization via gel entrapment techniques is widely utilized in biotechnology at a laboratory level and also in some selected cases on an industrial scale (Brodelius *et al.*, 1987). However, gel carriers are the hindrance of substrate diffusion to the immobilized cells and of metabolites leaving the gel-entrapped biomass (Maryse *et al.*, 1996). In order to reduce such carrier-induced additional diffusion barriers, highly porous matrices are, therefore, necessary. Another main factor that influences the immobilization behaviour of the cells and their productivity is thought to be the surface characteristics of the carrier including pore size, hydrophilicity and magnetism (Shinonaga *et al.*, 1992; Passarinho *et al.*, 1989).

The benefits of the immobilization process in microbial fermentations are as follows (Kargi, 2001; Karagoz *et al.*, 2008):

- ❖ Produces high cell concentrations that maintain high productivity throughout the operation period, and avoid wash out during high dilution rate.
- ❖ Provides protection for the cells from the harsh environment and is highly beneficial to shear sensitive organisms when operating at high dilution rates.
- ❖ The supports are stable and resistant to extreme environments such as pH, temperature, and toxic metabolites.